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14. ABSTRACT

Our ***long-term goal*** is to understand the impact of high levels of SNAI repressor proteins in the etiology, progression and pathogenicity of breast cancer. Our ***central hypothesis*** is that combinatorial treatment of SNAI-high breast tumor cells with the SNAI inhibitors will not only diminish their aggressiveness but also make these cells sensitive to the inhibition of some of the conventional anticancer agents such as vitamin D and anti-estrogens. **Specific Aims: (1)** To evaluate the levels of SNAI proteins in relation to the levels of the proteins that impart resistance against vitamin D and anti-estrogens in human breast cancer tissues; **(2)** To determine the effects of alterations of the levels of SNAI proteins in breast cancer cells on their sensitivity towards vitamin D and/or 4HT; and **(3)** To evaluate the efficacy of RNA and peptide-based inhibitors of SNAI-protein functions on the aggressiveness, metastatic ability and drug sensitivity of SNAI-high human breast cancer cells in a mouse xenograft model.

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INTRODUCTION

Breast cancer is the most prevalent cancer in women [1]. Because of the complexity and heterogeneity of mammary carcinogenesis [2], many pharmacologic agents have been studied for their effects on the prevention of breast cancer including selective ER modulators [3, 4]. However, these drugs are not effective in preventing ER-negative breast cancer, which corresponds to at least one third of the breast cancer cases [5]. The vitamin D receptor (VDR), a member of the nuclear receptor superfamily, has been suggested as a target for both ER-positive and ER-negative breast cancer prevention [6-12] because it is not deleted in most breast tumors [5], and VDR ablation in mice was reported to enhance carcinogen induced formation of mammary tumors [13]. These results suggest a role of vitamin D signaling in the regulation of mammary tumorigenesis. One problem in targeting VDR in breast cancer therapy is that VDR is often down regulated in many aggressive and invasive breast tumors [6-12]. We propose that the efficacy of vitamin D therapy will depend upon the level of the transcriptional repressor of SNAI family in the human breast tumor cells. The research proposed here will evaluate this notion.

Estrogen receptor alpha (ERα)-positive breast cancer patients are commonly treated with either aromatase inhibitors or anti-estrogens [14-17]. The clinically most frequently applied anti-estrogen, 4-hydroxy-tamoxifen (4HT), leads in an adjuvant setting to an approximately 50% reduction in recurrence during 10 years of follow-up of ER-positive patients, and to a decrease in mortality by a third [18-20]. The effectiveness of anti-hormone therapy compels the pharmaceutical industry to continuously innovate new pharmacologically improved anti-estrogen SERMs and SERDs [21-24]. Many factors may affect clinical response to new anti-estrogens, including overexpression of cyclin D1 [25-27]. Over expression of cyclin D1, however, resulted in a conformational arrest of ERα, activating ERα, but now in the presence of 4HT [24, 28]. In this conformation, the ERα is capable of recruiting RNA polymerase II and inducing chromatin remodeling required for initiation of transcription [25-27]. There is an urgent need to know how tumors evade therapy and how they become resistant. Our notion, based on our recently published data [29], is that SNAI-high ER-positive breast cancer cells have higher levels of cyclin D1 due to the repression of the UbcH5c, a negative regulator for cyclin D1, by SNAI2 and thus they are 4HT-resistant [29]. Inhibition of SNAI proteins should bring back 4HT sensitivity [29].

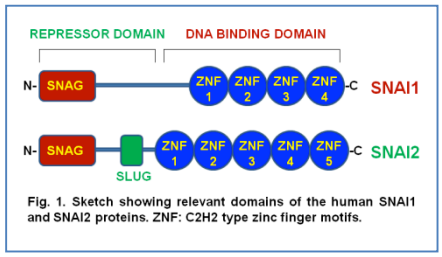


Fig. 1. Sketch showing relevant domains of the human SNAI1 and SNAI2 proteins. ZNF: C2H2 type zinc finger motifs.

The focus of our proposed research is on two members of the SNAI superfamily of zinc-finger transcriptional repressors: SNAI1 (also known as SNAIL) and SNAI2 (also known as SLUG) [30-34]. Human SNAI proteins encode C_2H_2-type zinc finger transcription factors that bind to E2-box motif (5′-CAGGTG-3′/5′-CACCTG-3′), and silence gene expressions by chromatin remodeling [30-35]. SNAI1

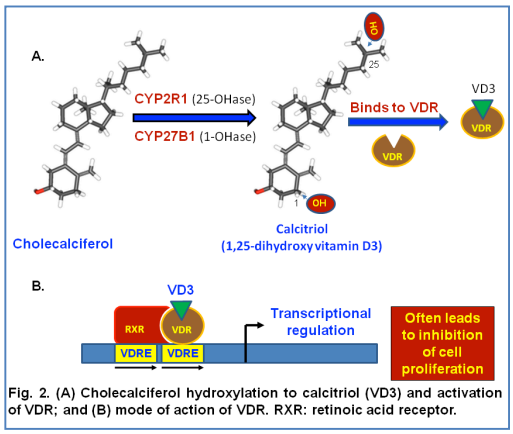


Fig. 2. (A) Cholecalciferol hydroxylation to calcitriol (VD3) and activation of VDR; and (B) mode of action of VDR. RXR: retinoic acid receptor.

and SNAI2 are very similar in their amino acid sequences at the C-terminal zinc-finger domains, but the N-terminal repressor domain is somewhat different (Fig. 1).

Vitamin D plays roles in various physiological processes, including bone and calcium metabolism, cellular growth and differentiation, immunity, and cardiovascular function [36-39]. Vitamin D is synthesized from 7-dehydrocholesterol, an intermediate metabolite in cholesterol synthesis, or derived from dietary sources [40-42]. Ultraviolet irradiation in sunlight-exposed skin induces a photochemical reaction of 7-dehydrocholesterol to produce the vitamin D_3 (cholecalciferol). Vitamin D_3 is hydroxylated at the 25-position by vitamin D 25-hydroxylase (CYP2R1), to yield 25-hydroxyvitamin D_3 ($25(OH)D_3$; 25-hydroxycholecalciferol), the major form of vitamin D in the circulation (Fig. 2). $25(OH)D_3$ is further hydroxylated at the 1α-position by 25-hydroxyvitamin D 1a-hydroxylase (CYP27B1) (Fig. 2). VD3 exhibits physiological and pharmacological effects by binding to the vitamin D receptor (VDR), a transcription factor of the nuclear receptor superfamily [40-42] (Fig. 2). Although photoactivated cholecalciferol is mainly hydroxylated in liver and kidney, many other cells including breast cells have significant expressions of CYP2R1, CYP27B1 and VDR [40-42]. Breast cells thus should be able to activate and utilize cholecalciferol if these proteins are not suppressed. We have recently found that SNAI proteins coordinately repress the transcriptions of CYP2R1, CYP27B1 and VDR, but not those of CYP24A1, GC and RXR, in several human cell lines [43] (see below). Because low levels of CYP2R1, CYP27B1 and VDR is associated with vitamin D resistance [44-46], and because SNAI proteins can decrease the expression of CYP2R1, CYP27B1 and VDR [43, 47], we propose to test the novel hypothesis that high levels of SNAI proteins in cells can reduce the therapeutic efficacy of vitamin D by reducing the production of CYP2R1, CYP27B1 and VDR.

Our ***long-term goal*** is to understand the impact of high levels of SNAI repressor proteins in the etiology, progression and pathogenicity of breast cancer. The ***objective for this IDEA expansion proposal*** is to develop and evaluate the efficacy of nucleic acid- and peptide-based combinatorial drug regimen against the functions of both of the SNAI proteins to antagonize the growth and metastasis of SNAI-high breast tumors that are resistant to vitamin D and/or the anti-estrogen 4-hydroxy tamoxifen (4HT). Our ***central hypothesis*** is that combinatorial treatment of SNAI-high breast tumor cells with the SNAI inhibitors will not only diminish their aggressiveness but also make these cells sensitive to the inhibition of some of the conventional anticancer agents such as vitamin D and anti-estrogens.

Specific Aims to test the hypothesis are: (1) To evaluate the levels of SNAI proteins in relation to the levels of the proteins that impart resistance against vitamin D and anti-estrogens in human breast cancer tissues; **(2)** To determine the effects of alterations of the levels of SNAI proteins in breast cancer cells on their sensitivity towards vitamin D and/or 4HT; and **(3)** To evaluate the efficacy of RNA and peptide-based inhibitors of SNAI-protein functions on the aggressiveness, metastatic ability and drug sensitivity of SNAI-high human breast cancer cells in a mouse xenograft model.

BODY

Relevant research data generated leading to the project. We have verified and refined most of these data during the current project period.

1. VDR gene expression is regulated by SNAI2 [19]: SNAI2 regulates the VDR gene promoter (-613 to +15) that contains three E2-box sequences (CAGGTG/CACCTG), the classical binding site of SNAI2 [46]. SNAI2 specifically inhibited VDR gene promoter activity. Chromatin-immunoprecipitation (ChIP) assays revealed that SNAI2 is recruited on the native VDR gene promoter along with the co-repressor protein CtBP1 and the effector protein HDAC1 [46]. These data suggests that SNAI2 binds to the E2-box sequences of the VDR gene promoter and recruits CtBP1 and HDAC1, which results in the inhibition of VDR gene expression by chromatin remodeling [46]. Our additional analysis of several human breast cancer cell lines indicated direct correlation among VDR level and VD3 sensitivity of the cells (Table 1). Of particular relevance to this proposal is our observation that expression of SNAI2 in SNAI2-negative breast cancer cells (MCF7 and MDA-MB-468) or siRNA-mediated knockdown of SNAI2 in SNAI2-positive cells (MDA-MB-231 and BT549) altered the sensitivity of these cells to VD3, as expected (Table 1).

Table 1. SNAI2 and VDR levels and VD3-sensitivity of different human breast cancer cell lines .

Cell Line	SNAI2 level	VDR level	Sensitivity to VD3 (100 nM)*	Cell Line	SNAI2 level	VDR level	Sensitivity to VD3 (100 nM)*
MDA-MB-231	++++	-	R	MCF7/SNAI2	++++	-	R
BT549	++++	-	R	468/SNAI2	++++	-	R
Hs578T	+++	-	R	549/SNAI2KD	+	+++	S
MDA-MB-468	+	++	S	231/SNAI2KD	+	+++	S
MDA-MB-175	-	+++	S	AU565	-	+++	S
MCF7	-	+++	S	HCC1806	+++	+	R
MDA-MB-436	+++	-	R	HCC70	+++	-	R
ZR-75-1	-	+++	S	UACC812	-	+++	S
T47D	-	+++	S	HCC1937	+	+++	S
BT474	-	+++	S	HCC1954	+	+++	S
SKBR3	-	+++	S	ZR-75-30	-	+++	S

*R: resistant; S: Sensitive

2. Breast cancer tissues/cells expressing SNAI proteins are low in VDR and are resistant to VD3. In a preliminary study, we recently evaluated the levels of SNAI1 or SNAI2 and, cyclin D1 and VDR in normal and tumor tissue of breast cancer patients by tissue microarray analysis (http://www.biomax.us/tissue-arrays/Breast/) [see ref. 29]. Variable expressions of these genes was detected in all 60 matched tumor and normal tissues studied. SNAI2 was expressed at higher level in tumor *versus* normal tissue (T:N>2.0) in 71% of patients, while *VDR* was down regulated (T:N<0.3) in 69%. Consistently with data obtained in cultured cells, we found a statistically significant inverse correlation between SNAI2 and VDR levels (Spearman correlation coefficient r = -0.3, p = 0.002). We also analyzed SNAI1 expression in the same biopsies. SNAI1 expression was found in 62% of tumors and it correlated with *VDR* down regulation (Kruskal-Wallis test, p<0.001). We will perform such studies with larger samples (200-300) in this proposal.

Table 2. Genes that are directly regulated by SNAI2 in human breast cancer cells.

Gene Name	Enriched/ control	Gene Name	Enriched/ control	Gene Name	Enriched/ control
*KRT8	6.2	ITGA3	4.7	PGDH	5.0
KRT18	5.9	PUMA	4.5	PALB2	4.9
KRT19	5.8	BRCA2	4.3	DCTN5	4.9
*CDH1	5.6	*CLDN1	4.2	SNAI1	4.4
OCLN	5.4	CLDN3	4.1	JUP	3.7
BAX	5.4	CLDN5	4.1	UbE2D3	3.7
*VDR	5.3	*CLDN7	4.1	MTCH2	3.4
DSG1	5.2	CLDN11	4.0	BicD1	5.1
DSG2	5.1	*CYP2R1	4.2	CAV1	5.2
PCNA	5.1	*CYP27B1	3.3	BCAS4	4.8

*Genes are also repressed by SNAI1.

3. ChIP-DSL analysis suggests several targets for SNAI2 including VDR, CYP2R1 and CYP27B1. We recently determined the gene promoters that bind to SNAI2 in human breast cancer cells by promoter array analysis following ChIP-DSL (Aviva) techniques [29]. We further validated the regulations of 30 of those genes by SNAI2 in several human breast cancer cells through qRT-PCR, Western blotting and promoter activity analyses (see Table 2). We found some of those genes are co-regulated by SNAI1 (Table 2). This analysis and further detailed

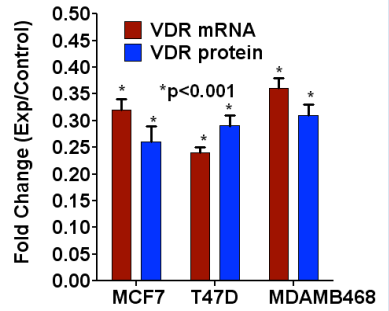


Fig. 3. Evaluations of VDR mRNA (red) and protein (blue) levels in VDR knocked down human breast cancer cells. Cells treated with mutated siRNA was used as control. Results are mean ± SEM (n=4).

analysis (communicated) reveal that SNAI2 represses the expression of SNAI1 in human breast cells. This observation is particularly significant for the current proposal suggesting that we must down regulate both SNAI1 and SNAI2 simultaneously to achieve de-repression of the target genes such as VDR. Three other major cholecalciferol metabolizing proteins, GC (vitamin D binding transport protein; ref. 66), CYP24A1 and RXR, were not repressed by the SNAI proteins. Their gene promoters also did not bind to the SNAI proteins in the cell lines tested.

4. Knockdown of VDR in cells decreased their sensitivity to VD3. We evaluated further whether knockdown of VDR in the SNAI-low VDR-high cells will make these VD3-sensitive cells resistant to VD3. We knocked down VDR in these cells using Stealth siRNAs designed against human VDR mRNA (Invitrogen). Both VDR mRNA and protein levels were reduced (70-80%) by the Stealth siRNA treatment (Fig. 3). VDR mRNA levels were determined by real-time RT-PCR using 18S rRNA as control. VDR protein level was determined by Western blotting. Bands were developed using donkey anti-rabbit IR Dye 800 (LI-COR biosciences) secondary antibody, and visualized using LI-COR's Odyssey Infrared Imaging System. Quantitation and analysis of bands were performed using Odyssey's software [29]. The VDR knocked down cells became VD3 resistance (Fig. 4), verifying our hypothesis. SNAI-high/VDR-low MDA-MB-231 cells were used as control (Fig. 4).

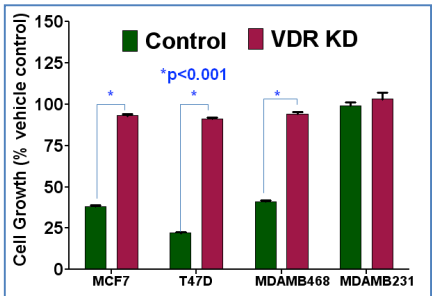


Fig. 4. Development of resistance to VD3 (10 nM, 72 h) due to knockdown of VDR in different human breast cancer cells. Growth was determined by ^{3}H-thymidine incorporation assay. Results are mean ± SEM (n=4). MDA-MB-231 cells do not express VDR and are thus naturally VD3 resistant.

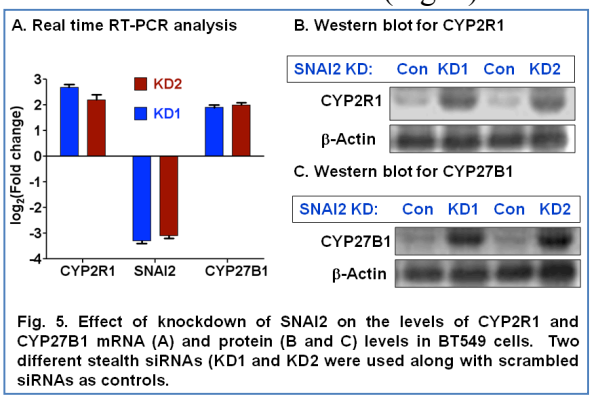


Fig. 5. Effect of knockdown of SNAI2 on the levels of CYP2R1 and CYP27B1 mRNA (A) and protein (B and C) levels in BT549 cells. Two different stealth siRNAs (KD1 and KD2 were used along with scrambled siRNAs as controls.

5. We validated that SNAI2 directly inhibits the expressions of the vitamin D activating enzymes CYP2R1 and CYP27B1 in human cells. We evaluated CYP2R1 and CYP27B1 levels in the SNAI2 knocked down cells; we found that CYP2R1 and CYP27B1 mRNA and protein levels are increased significantly in these cells (Fig. 5). We found that the promoters of human CYP2R1 and CYP27B1 genes have at least one potential SNAI2 binding E2-box. We cloned these promoters in front of *Renilla* luciferase gene in pRL-Null vector and evaluated the promoter activities with or without knock down of SNAI2 mRNA in the BT549 cells. As expected, CYP2R1 and CYP27B1 promoter activities went up when SNAI2 mRNA was knocked down (Fig. 6). We also have shown by ChIP assay that SNAI2 is specifically recruited at the CYP2R1 and CYP27B1 gene promoters in the BT549 cells (Fig. 6). Similar observations were made with SNAI1 knocked down BT549 cells. This observation strongly suggests that SNAI proteins do not only prevent the function of VD3 by repressing VDR gene expression but also prevent the activation of vitamin D through the repression of its hydroxylases CYP2R1 and CYP27B1.

6. We successfully adopted a sensitive assay system for VDR function in human cells. We verified that VD3 (calcitriol) exerts growth inhibitory effects on VDR-positive breast cancer cells (Table 1) by inducing G0/G1 cell cycle arrest and apoptosis as was demonstrated by others for tumor-derived epithelial cells. Upon treatment with calcitriol, VDR expression was induced in VDR-positive cells but not in the VDR-negative (Table 1) human breast cancer cells. We successfully adopted using the 24-hydroxylase promoter-luciferase reporter [67] to evaluate the transactivation of CYP24, a calcitriol responsive gene [68]. We demonstrated that in cells expressing VDR, but not those without VDR, calcitriol transactivated CYP24. This method will be useful for our studies to evaluate the activity of VD3 and VDR in breast cancer cells.

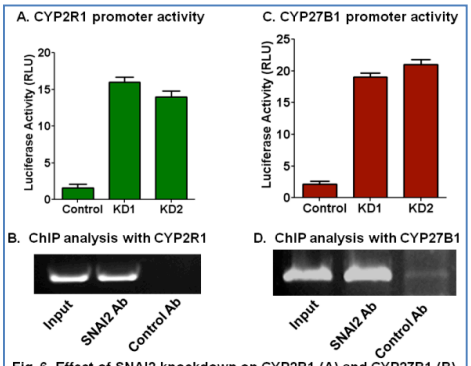


Fig. 6. Effect of SNAI2 knockdown on CYP2R1 (A) and CYP27B1 (B) promoter activities. Figs. C and D show *in vivo* bindings of SNAI2 to these promoters.

7. We found that SNAI2 inhibits the expression of SNAI1 in human cells. Our recent studies with BT549 cells showed that when we knocked down SNAI2 with siRNA, SNAI2 levels goes down more than 80% but some of the SNAI2 target genes are not de-repressed accordingly. When we evaluated SNAI1 levels in the SNAI2 knocked down cells, surprisingly we found that SNAI1 mRNA and protein levels are increased significantly in these cells (Fig. 7). We found that the promoter of human SNAI1 gene has four potential SNAI2 binding E2-boxes (Fig. 8A, shown in color). We cloned this promoter in front of *Renilla* luciferase gene in pRL-Null vector and evaluated the promoter activities with or without knock down of SNAI2 mRNA in the BT549 cells. As expected, SNAI1 promoter activity went up when SNAI2 mRNA was knocked down (Fig. 8B). We also have shown by ChIP assay that SNAI2 is specifically recruited at the SNAI1 gene promoter in the BT549 cells (Fig. 8C). This observation is significant with respect to the current proposal because these data suggest that we must knockdown SNAI1 and SNAI2 together to achieve de-repression of target genes such as CYP2R1, CYP27B1 and VDR.

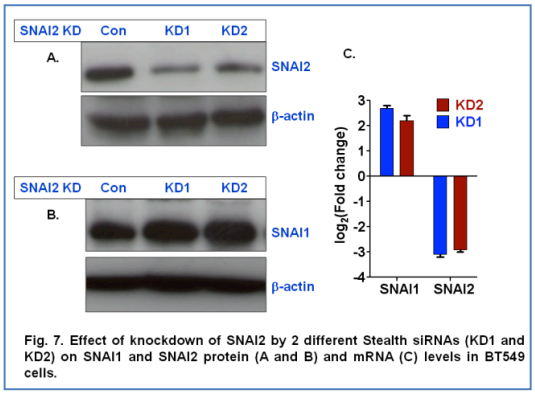


Fig. 7. Effect of knockdown of SNAI2 by 2 different Stealth siRNAs (KD1 and KD2) on SNAI1 and SNAI2 protein (A and B) and mRNA (C) levels in BT549 cells.

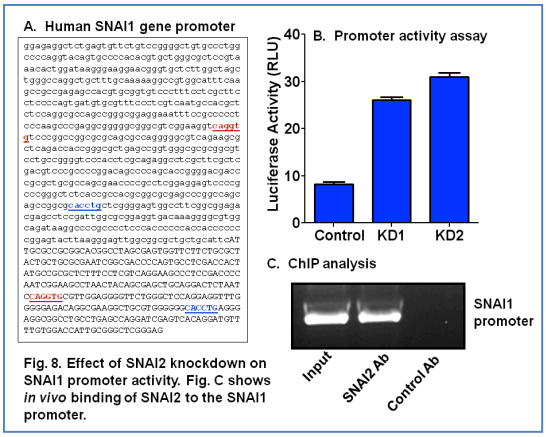


Fig. 8. Effect of SNAI2 knockdown on SNAI1 promoter activity. Fig. C shows *in vivo* binding of SNAI2 to the SNAI1 promoter.

8. SNAI2 overexpression elevated the level of cyclin D1 protein in breast cancer cells through the repression of the ubiquitination of this protein. We found [29] that SNAI2 inhibits the expression of *UbcH5c* directly through chromatin remodeling and thus, among other downstream effects, elevates the level of cyclin D1, enhancing the growth rates of breast cancer cells [29] (please see the accompanying reprint for experimental details). Overexpression of SNAI2 in the SNAI2-deficient breast cancer cells significantly decreased the levels of mRNA and protein of UbcH5c but only elevated the protein levels of cyclin D1 [29]. On the contrary, knockdown of SNAI2 in

SNAI2-high breast cancer cells elevated the levels of UbcH5c while decreasing the level of cyclin D1 protein [29]. SNAI2 is recruited at the E2-box sequence at the *UbcH5c* gene promoter along with the corepressor CtBP1 and the effector HDAC1 to silence the expression of this gene [29]. Knockdown of *UbcH5c* in the SNAI2-deficient human breast cells elevated the level of cyclin D1 as well as the rates of proliferation and invasiveness of these cells [29]. While the growth rates of the cells are enhanced due to overexpression of SNAI2 or knockdown of UbcH5c in the breast cancer cells tested, ER+ cells also acquire resistance to the anti-estrogen 4-hydroxytamoxifen due to the rise of cyclin D1 levels in these cells [29]. This study thus implicates high levels of SNAI2 and low levels of UbcH5c as a determinant in the progression of metastatic breast cancer [29].

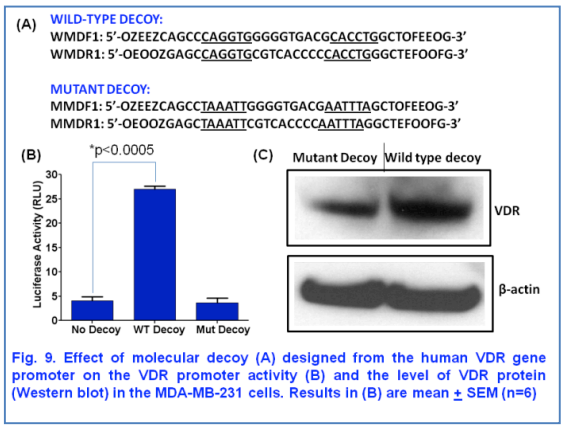


Fig. 9. Effect of molecular decoy (A) designed from the human VDR gene promoter on the VDR promoter activity (B) and the level of VDR protein (Western blot) in the MDA-MB-231 cells. Results in (B) are mean ± SEM (n=6)

9. Double-stranded DNA decoy designed against SNAI2 binding inhibited SNAI2 activity. We designed phosphorothioate gapmers from human VDR gene promoter by annealing two synthetic oligonucleotides (custom synthesized and gel purified from Invitrogen). This double-stranded oligomer (decoy) should be resistant to exonuclease digestion [69, 70] and should bind to SNAI2 (may also to SNAI1 as well) when introduced inside breast cells by oligofection (Invitrogen). We also designed similar molecular decoys with mutations in the E2-box sequences (Fig. 9A). We used this mutated decoy as control. As expected, when transfected into MDA-MB-231 cells at a concentration of 25 nM for 24 h, the wild-type decoy enhanced VDR promoter activity 6-7 folds (Fig. 9B) and the levels of VDR protein was increased significantly (Fig. 9C). We propose here that combinatorial therapy with SNAI protein inhibitors and VD3/4HT will inhibit the growth and proliferation of many breast cancers aberrantly over expressing SNAI proteins.

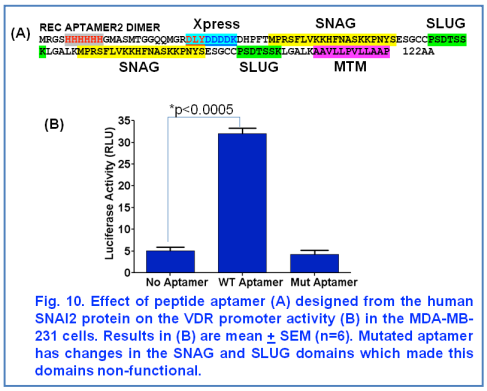


Fig. 10. Effect of peptide aptamer (A) designed from the human SNAI2 protein on the VDR promoter activity (B) in the MDA-MB-231 cells. Results in (B) are mean ± SEM (n=6). Mutated aptamer has changes in the SNAG and SLUG domains which made this domains non-functional.

10. Cell permeable protein aptamer developed against SNAI protein functions inhibited SNAI-mediated repression of VDR gene expression. We also have developed a recombinant protein containing dimer of SNAG and SLUG domains of SNAI2 (see Fig. 1) and a membrane translocation motif (MTM; AAVLLPVLLAAP) for the protein to be cell penetrable [71, 72] (Fig. 10A). We expressed and purified this protein from *E. coli* (Invitrogen). When delivered (1 μM, 24 h) to MDA-MB-231 cells, this aptamer significantly stimulated the VDR promoter activity (Fig. 10B), suggesting inhibition of SNAI protein function by this aptamer. The mode of action of this aptamer protein is not know and is the subject of the current proposal.

11. We adopted the 'wrapsome' technique for the delivery of nucleic acid and peptide-based combinatorial drugs to the breast tumor cells *in vitro* and *in vivo*. We packaged the nucleic acid and peptide-based SNAI2 inhibitors inside wrapsome (please refer to ref. 73 and SOW section for experimental details). We evaluated the efficacy of these drug formulations in tissue culture model as well as mouse xenograft model. Three week-old female

BALB/c nu/nu mice were used. Mice received irradiated normal rodent chow (Purina Test Diets, Richmond, IN). For inoculation into nude mice, $5x10^6$ MDA-MB-231 cells (control wrapsome-treated or SNAI2 inhibitor containing wrapsome-treated) were injected *s.c.* into the flank region and allowed to grow for two weeks. Tumor size was monitored twice weekly by caliper measurement of the length, width and height, and tumor volume was calculated using the formula for a semi-ellipsoid ($4/3\pi r^3/2$). After two weeks, mice with tumor volume averaging approximately 200 mm^3 (range 100-400 mm^3) were used for study. Tumor-bearing mice were then randomized into four groups (5 mice per group): (a) Control for wrapsome containing control nucleic acid and peptides but no VD3; (b) Control for wrapsome containing control nucleic acid and peptides with 0.3 mg/kg VD3; (c) Experimental for wrapsome containing SNAI2-inhibitory nucleic acid and peptides but no VD3; and (d) Experimental for wrapsome containing SNAI2-inhibitory nucleic acid and peptides with 0.3 mg/kg VD3. VD3 was dissolved in 80% propylene glycol/20% PBS and incorporated into the wrapsome during its formation. The wrapsomes were administered three times weekly *via* intraperitoneal injection. After five weeks of treatment, tumors were removed, weighed and fixed in 4% formalin for histological analysis. Tumor size and weight did not change by vitamin D treatment when control wrapsomes were used. On the other hand animals treated with SNAI2 inhibitor containing wrapsome decreased the size and weight of the tumor significantly, particularly when treated with vitamin D. Tumor volume decreased from 189 $\pm$ 17 mm3 to 42 $\pm$ 6 mm3 (n=5). Tumor weight was decreased from 120 $\pm$ 13 mg to 24 $\pm$ 3 mg (n=5). These data indicate the 'proof of concept' of our proposed studies.

Summary of the data generated during the expansion project period.

Task-1. To determine the potential of high levels of SNAI and cyclin D1 proteins and low levels of UbcH5c, CYP2R1, CYP27B1 and VDR proteins as biomarkers of aggressive breast cancers in breast cancer patients.

a. We procured human tissue samples from Meharry Medical College Tissue acquisition sources, Vanderbilt University Tissue acquisition sources and commercial sources to obtain Tissue Microarrays (TMAs) which include normal tissue from mammoplasty, normal tissue adjacent to primary tumor, atypical tissue from benign neoplasm, ductal carcinoma in situ (DCIS, non-invasive malignant tumor), estrogen receptor-positive invasive ductal carcinoma, HER2 positive invasive ductal carcinoma and triple-negative invasive ductal carcinoma. We used more than 150 samples.

b. We evaluated SNAI1, SNAI2, CYP2R1, CYP27B1, cyclin D1, UbcH5c and VDR expression in the TMAs. We used the above TMAs to analyze the levels these proteins under the same staining conditions.

Tissue microarrays from 190 primary invasive breast carcinoma patients (54 ER-positive, 16 HER-2/neu over-expressing and 120 triple-negative [50 caucasian and 70 African American]) were interrogated for the expression of these proteins by immunohistochemical analysis. We found ~80% of the triple negative breast tumors have higher levels of SLUG and lower levels of SNAIL, CYP2R1, CYP27B1, UbcH5c and VDR (Table 3). The level of cycline D1 is proportional with the SLUG levels (Table 3). Fig. 11 summarizes our model for inhibition of growth of SLUG-high triple negative breast cancer cells by combinatorial treatment with SLUG inhibitor and Vitamin D.

Table 3. Correlation among SNAI1, SNAI2, CYP2R1, CYP27B1, cyclin D1, UbcH5c and VDR expression in the TNBC TMAs.

	VDR	CYP2R1	CYP27B1	Cyclin D1	UbcH5c
SNAI2-low CA=13 AA=11	CA= H:10 AA= H:11	CA= H:10 AA= H:11	CA= H:10 AA= H:10	CA= L:8 AA= L:9	CA= H:10 AA= H:11
SNAI2-high CA=37 AA=59	CA= L:32 AA= L:58	CA= L:30 AA= L:58	CA= L:31 AA= L:56	CA= H:37 AA= H:59	CA= L:36 AA= L:56

CA= Caucasian American; AA= African American; H= high; L= low.

Task-2. To optimize dsDNA decoys and peptide aptamers developed against the functions of SNAI proteins. The decoy oligodeoxynucleotide (ODN) strategy to control the activity of a transcription factor (TF) involves transfection of double-stranded (ds) ODNs, the sequence of which corresponds to the binding site sequence of the TF in question. These ODNs can compete for TF binding with the native TF binding site, thus leading to the inhibition of downstream gene regulation by the targeted TF [59-61]. We knocked down SNAI1 and/or SNAI2 activities in the SNAI1 and/or SNAI2-positve cells using chimeric ds-DNA decoy [62, 63] and/or peptide aptamer [64, 65]. Double-stranded SNAI1 and SNAI2 decoy ODNs were synthesized according to the cis-element sequence of the binding site of these proteins on 8-10 different SNAI target gene promoters. The E2-box mutated ODNs (MM) were used as negative controls. 3′ and 5′ phosphorothioate modifications were added to the ODNs to enhance their stability. The 5′ terminus of a subset ODNs were labeled with FITC to identify gene distribution under fluorescence microscopy. EMSA were performed to confirm that the SNAI protein decoy ODN

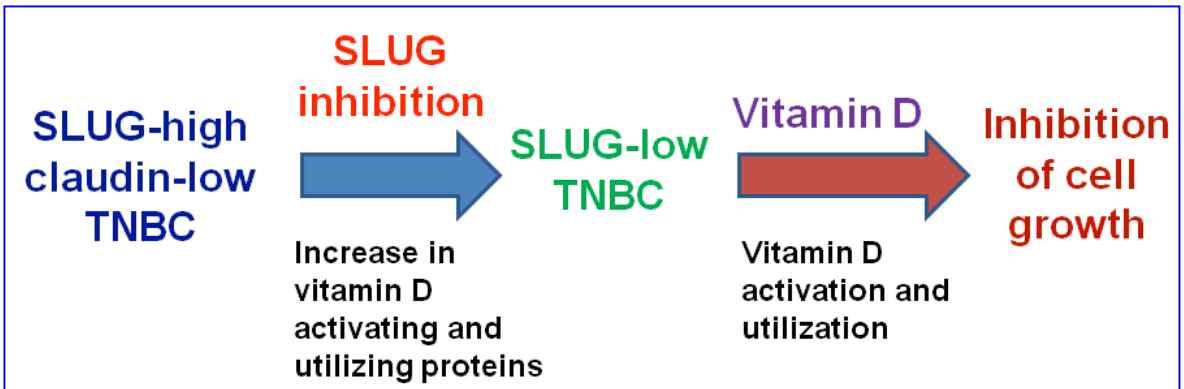


Fig. 11. Model for inhibition of growth of SLUG-high triple negative breast cancer cells by combinatorial treatment with SLUG inhibitor and Vitamin D.

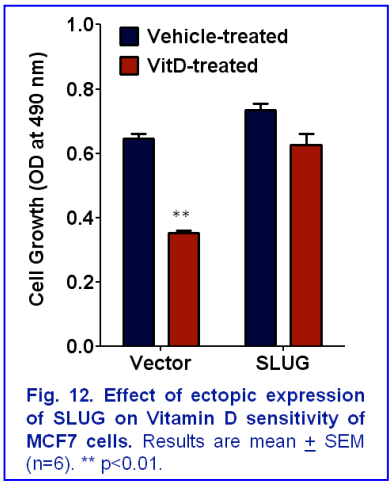


Fig. 12. Effect of ectopic expression of SLUG on Vitamin D sensitivity of MCF7 cells. Results are mean ± SEM (n=6). ** p<0.01.

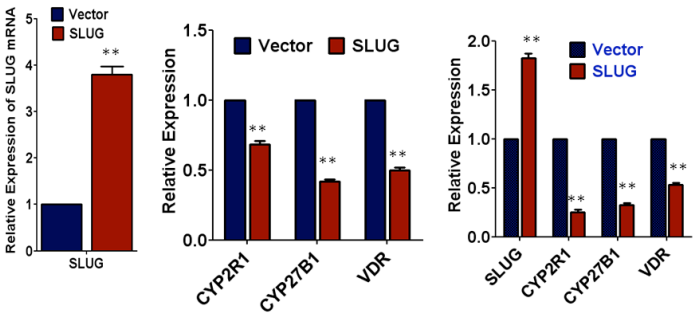


Fig. 13. Effect of ectopic expression of SLUG on the levels of different mRNAs in SLUG-negative MCF7 (A and B) and T47D cells. Results are mean ± SEM (n=4). ** p<0.01.

binds to the respective SNAI protein in the breast cancer cells. SNAI binding was assessed by the chemiluminescent EMSA using biotin-labeled ODN as probe. Fig. 12 presents the effect of ectopic expression of SLUG on Vitamin D sensitivity of SLUG-negative MCF7 cells. Fig 13 summarizes the effect of ectopic expression of SLUG on the levels of different mRNAs in SLUG-negative MCF7 (A and B) and T47D cells. We designed phosphorothioate gapmers from human VDR (a SLUG target gene) gene promoter by annealing two synthetic oligonucleotides (Invitrogen). This dsDNA was resistant to exonuclease digestion and binds to SLUG in EMSA analysis. Similar dsDNA was also made with mutations in the E2-box sequences to use as a negative control (Fig. 14). This dsDNA decoy was able to release SLUG target genes such as plakoglobin, from SLUG repression in the MDA-MB-231 cells (Fig. 15).

Task-3. To evaluate dsDNA decoys and peptide aptamers developed against the functions of SNAI proteins in tissue culture models. We selected and characterized 4 SNAI-high or

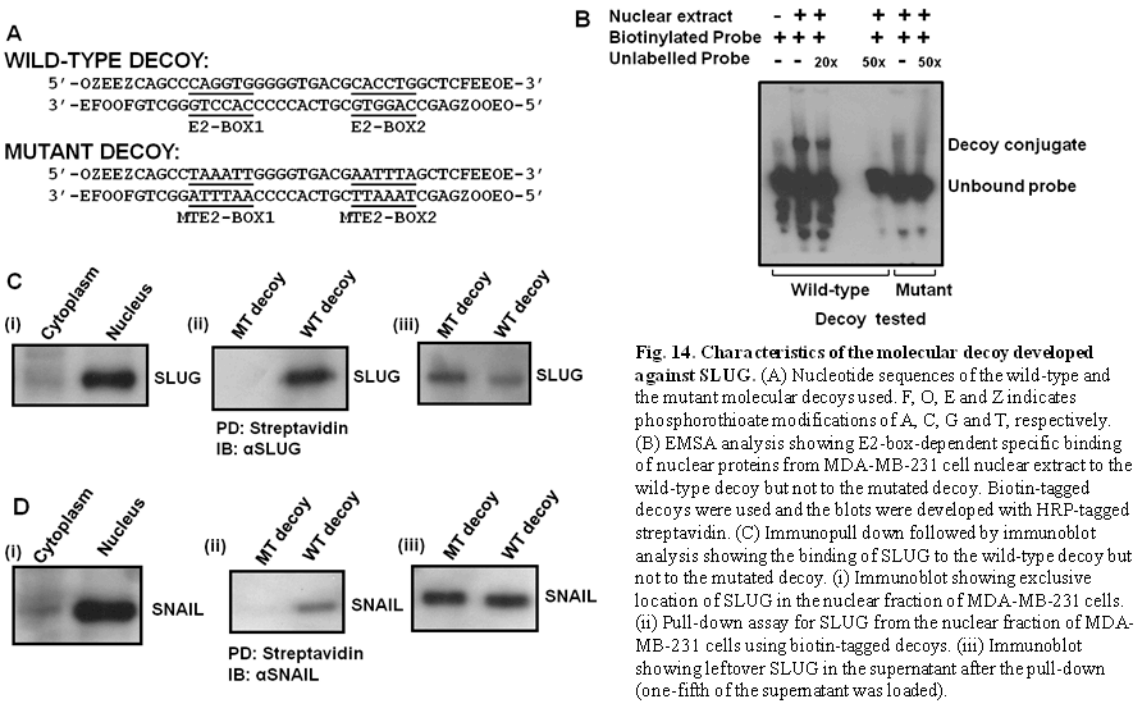
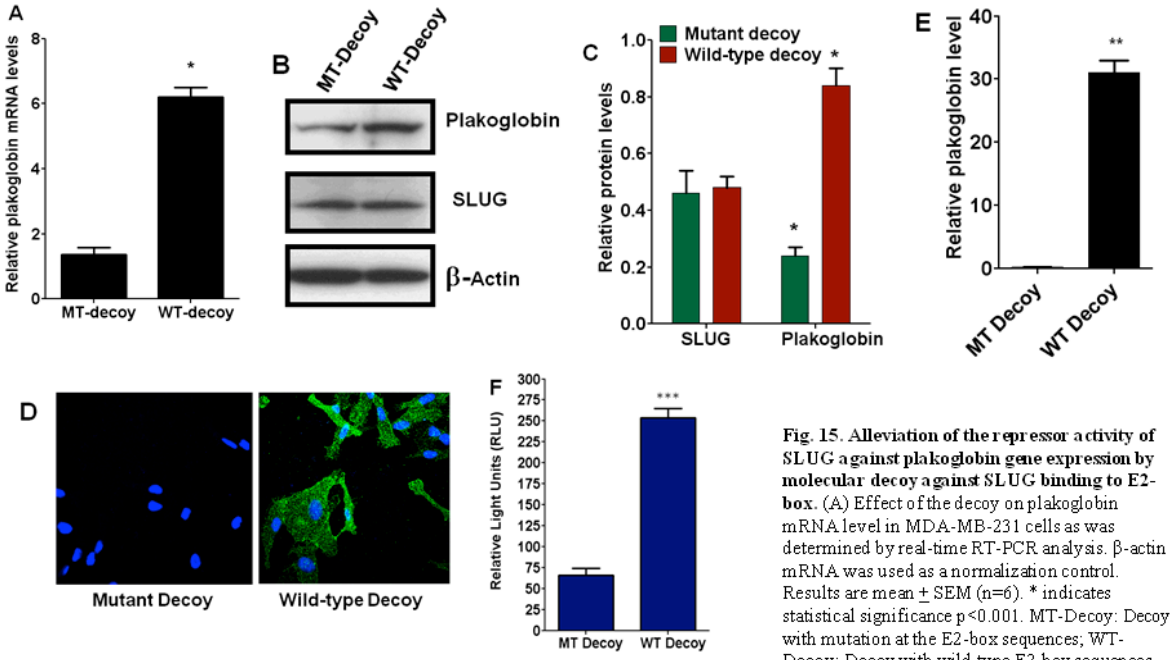


Fig. 14. Characteristics of the molecular decoy developed against SLUG. (A) Nucleotide sequences of the wild-type and the mutant molecular decoys used. F, O, E and Z indicates phosphorothioate modifications of A, C, G and T, respectively. (B) EMSA analysis showing E2-box-dependent specific binding of nuclear proteins from MDA-MB-231 cell nuclear extract to the wild-type decoy but not to the mutated decoy. Biotin-tagged decoys were used and the blots were developed with HRP-tagged streptavidin. (C) Immunopull down followed by immunoblot analysis showing the binding of SLUG to the wild-type decoy but not to the mutated decoy. (i) Immunoblot showing exclusive location of SLUG in the nuclear fraction of MDA-MB-231 cells. (ii) Pull-down assay for SLUG from the nuclear fraction of MDA-MB-231 cells using biotin-tagged decoys. (iii) Immunoblot showing leftover SLUG in the supernatant after the pull-down (one-fifth of the supernatant was loaded). (D) Immunopull down followed by immunoblot analysis showing weak binding of SNAIL to the decoy. (i) Immunoblot showing exclusive location of SNAIL in the nuclear fraction of MDA-MB-231 cells. (ii) Pull-down assay for SNAIL from the nuclear fraction of MDA-MB-231 cells using biotin-tagged decoys. (iii) Immunoblot showing leftover SNAIL in the supernatant after the pull-down (one-fifth of the supernatant was loaded).

SNAI-low breast cancer cell lines of African American and Caucasian origins (ICBP50, ATCC) for their expressions and functions of SNAI1, SNAI2, CYP2R1, CYP27B1, cyclin D1, UbcH5c and VDR. We over expressed SNAI1 and/or SNAI2 as C-terminal FLAG-tagged proteins in the SNAI1 and/or SNAI2-negative cells using our lentiviral stocks containing tetracycline-inducible expression constructs. Knockdown of SNAI1 and/or SNAI2 in the SNAI1 and/or SNAI2-positve cells was accomplished using siRNAs/shRNAs. We also knocked down SNAI1 and/or SNAI2 activities in the SNAI1 and/or SNAI2-positve cells using ds-DNA decoys. In addition, knockdown of SNAI1 and/or SNAI2 activities in the SNAI1 and/or SNAI2-positve cells was also accomplished using peptide aptamers and verified. We evaluated CYP2R1, CYP27B1,



(D) Immunofluorescence confocal microscopy analysis of the effect of the SLUG decoy on plakoglobin protein (green) levels in MDA-MB-231 cells. TOPRO (blue) was used to stain the nucleus of the cells. (E) Quantitative evaluation of the relative levels of plakoglobin (with respect to TOPRO staining) in the immunofluorescence images as in (D). Results are mean ± SEM (n=7). The difference in the relative (normalized with TOPRO level) plakoglobin levels between the cells treated with mutant decoy (MT Decoy) and wild-type Decoy (WT Decoy) was statistically significant. ** indicates statistical significance $p<0.01$. (F) Effect of the decoy on the activity of plakoglobin gene promoter in MDA-MB-231 cells. Results are mean ± SEM (n=6). *** indicates statistically significant changes between the control and the experimental sets ($p<0.05$).

Fig. 15. Alleviation of the repressor activity of SLUG against plakoglobin gene expression by molecular decoy against SLUG binding to E2-box. (A) Effect of the decoy on plakoglobin mRNA level in MDA-MB-231 cells as was determined by real-time RT-PCR analysis. β-actin mRNA was used as a normalization control. Results are mean ± SEM (n=6). * indicates statistical significance $p<0.001$. MT-Decoy: Decoy with mutation at the E2-box sequences; WT-Decoy: Decoy with wild-type E2-box sequences. (B) Western blot analysis showing the effect of the decoy on plakoglobin protein level in MDA-MB-231 cells. SLUG levels did not significantly alter. β-Actin was used as a loading control. (C) Densitometric scanning of the western blots to evaluate the relative levels of SLUG and plakoglobin in MDA-MB-231 cells. Results are mean ± SEM (n=4). * indicates statistical significance $p<0.001$.

cyclin D1, UbcH5c and VDR levels in the cells by real-time RT-PCR and Western blotting analyses. We also evaluated the doubling time and saturation density, cellular proliferation, anchorage-independent growth, cellular migration, cellular invasion, sensitivity to anoikis and perform 3D culture assays to analyze invasiveness, proliferation or matrix degradation. Data obtained from these experiments confirmed our initial experimental outcomes described above.

Task-4. Preparation of wrapsomes containing siRNA, purified peptide aptamer, ds-DNA decoy and VD3 and their characterization. Formulations of nucleic-acid and peptide-based cocktails (0-100 nM each) containing VD3 (0-100 nM) packaged in 'wrapsomes'. The package included siRNAs against HMGB proteins (HMGB1-3) to prevent induction of innate immune reactions by the nucleic acids in the target cells. We evaluated siRNA stability against nuclease by incubating 1 nmol of siRNA/WS or siRNA in saline solution with 0.2 IU RNase A (Qiagen).

The siRNA was then be extracted and separated on 15% TBE-urea gels (Invitrogen). Data obtained from these experiments confirmed our initial experimental outcomes described above.

Task-5. To evaluate the efficacies of combinatorial drug regimen that includes dsDNA decoys against the functions of SNAI proteins during VD3 treatment of experimental VD3-resistant breast cancer in mice xenograft model. We developmed highly invasive and metastatic SNAI1 and/or SNAI2-positve and CYP2R1, CYP27B1 and VDR-negative cells expressing either a luciferase gene or the red fluorescent protein dtTomato. We have MDA-MB-231 cells that are expressing either a luciferase gene or the red fluorescent protein dtTomato. We evaluated the combinatorial drug regimen in the highly invasive and metastatic SNAI1 and/or SNAI2-positve/cyclin D1-high and CYP2R1, CYP27B1, UbcH5c and VDR - negative cells. For each cell line we used female NOD/SCID mice. We analyzed a total of 4 cell lines. The number of mice needed for each experiment was determined by power analysis using the GraphPad STATMATE software. We evaluated the combinatorial drug regimen with different cell lines in orthotopic mouse models using the cells expressing either a luciferase gene or the red fluorescent protein dtTomato. The cell lines will be injected into the mammary fat pad of nude mice. The expression of luciferase or dtTomato will allow us to locate tumor cells and to measure tumor growth by utilizing *in vivo* imaging system. Figs. 16 and 17 summarizes our animal experiment data.

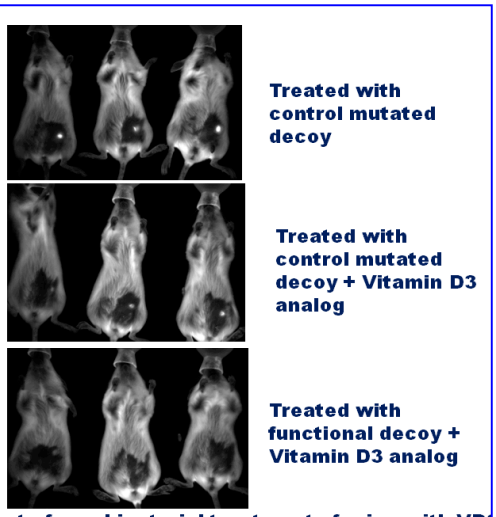


Fig. 16. Effect of combinatorial treatment of mice with VD3 dsDNA decoy for 4 weeks on tumor in mice.

Key Research Accomplishments

(1) We identified SNAI1, SNAI2, CYP2R1, CYP27B1, cyclin D1, UbcH5c and VDR expressions as breast epithelial markers, which are altered during the gradual progression from normal tissue to invasive breast cancer. This correlation will significantly advance the diagnosis of the invasive potential and aggressiveness of breast tumors.

(2) Our study revealed that the aggressiveness and severity of breast tumor development is determined at least in part by the ability of the tumor cells to respond to the endogenous VD3, decrease in the VDR levels will make the tumor cells resistant to endogenous VD3 thus leading to the malignancy. Since breast cancer in African American patients is often severe and aggressive with rapid fatal outcome, our study revealed SNAI protein up regulation and hence VDR gene promoter repression is one of major causes of such racial disparity in breast cancer.

(3) We have designed the SNAI protein knockdown and VDR activation strategy in a way that, once successfully tested in our preclinical studies, it can be subsequently used for clinical application. Nucleic acid-based drugs have promising prospects as future chemotherapeutic agents. Our research provided preclinical evidence that justifies phase I and II clinical trials, which will be planned and performed in the future.

(4) Our research revealed that dysregulated over production of SNAI proteins and thus down regulation of VDR is related to the racial disparity in breast cancer severity and progression**.** Endogenous VD3 protects our cells from over growth and thus conceivably prevents breast tumor cells from aggressively proliferating into malignant tumors. The black-to-white disparity in age-standardized breast cancer mortality is largely driven by the higher hazard rates of breast cancer death among black women, diagnosed with the disease, irrespective of ER expression, and especially in the first few years following diagnosis (*J. Natl. Cancer Inst.* 2009; **101**: 993-1000). Recent studies revealed that African American patients with breast cancers had worse survival than white patients, despite enrollment on phase III SWOG (Southwest Oncology Group) trials with uniform stage, treatment, and follow-up (*J. Natl. Cancer Inst*. 2009; **101**: 984-992). A complex set of genetic and environmental factors stringently regulate the expressions of the SNAI proteins. Our research found that disproportionate expression of the SNAI proteins and VDR knockdown is associated with breast cancer of the African Americans as compared to that of the Caucasian breast cancer patients.

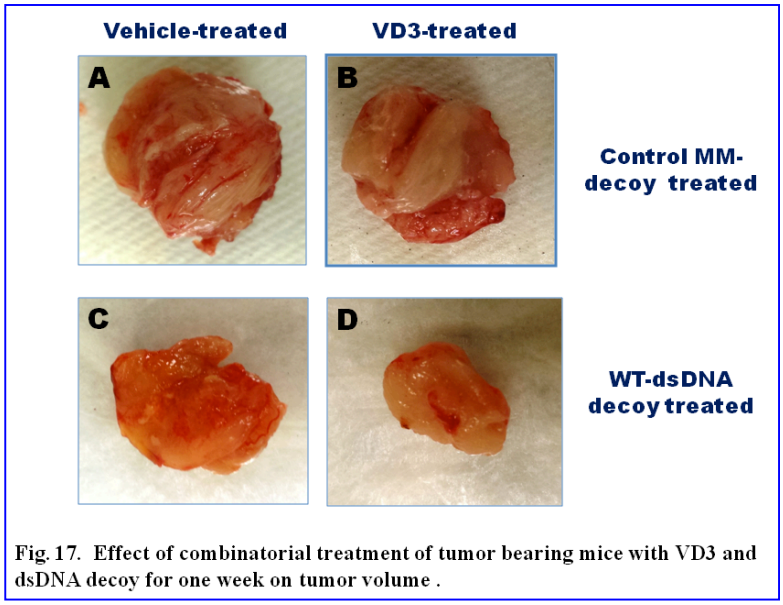


Fig. 17. Effect of combinatorial treatment of tumor bearing mice with VD3 and dsDNA decoy for one week on tumor volume .

(5) Our research investigated and revealed new therapeutic strategies. We used nucleic acid and protein based compounds for the knockdown of the activities of the SNAI proteins in the breast cancer cells. This minimized the possibilities of resistant development. We employed 'wrapsome' techniques to deliver the nucleic acid and protein based drug cocktail to the tumor cells in the animal model of breast cancer. This ensured increased sensitivity, less toxicity and fewer requirements of the drugs.

REPORTABLE OUTCOMES:

Publication:

1. Mittal M. K., Singh, K., Misra, S. and **Chaudhuri, G**. (2011) SLUG-induced elevation of D1 cyclin in breast cancer through the inhibition of its ubiquitination. ***J. Biol. Chem*. 286**, 469-479.

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3. Hall, M. 3rd, Misra, S., Chaudhuri, M., and **Chaudhuri, G.** (2011) Peptide aptamer mimicking RAD51-binding domain of BRCA2 inhibits DNA damage repair and survival in *Trypanosoma brucei*. ***Microb. Pathog***. **50,** 252-262.
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6. Bailey, C. K., Mittal, M. K., Misra, S., and **Chaudhuri, G**. (2012) High motility of triple-negative breast cancer cells is due to repression of plakoglobin gene by the metastasis modulator protein slug. ***J. Biol. Chem***. **287**, 19472-19486. PubMed PMID: 22496452.
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Meeting abstracts:

1. Cooper, R., Misra, S, Chaudhuri, M. and **Chaudhuri, G**. **(2012)** *Trypanosoma brucei*: A model to evaluate joint contribution of BRCA2 and PARP in DNA damage repair. **Presented at the AACR Annual Meeting in Chicago, IL, March 31 to April 4, 2012.**
2. Bailey, C. K., Mittal, M. K., Misra, S. and **Chaudhuri, G. (2012)** High mobility of triple-negative breast cancer cells is due to repression of plakoglobin gene by SLUG. **Presented at the AACR Annual Meeting in Chicago, IL, March 31 to April 4, 2012.**
3. Mittal, M. K., and **Chaudhuri, G. (2012)** Development and evaluation of a molecular decoy against SLUG function in the breast cancer cells. **Presented at the AACR Annual Meeting in Chicago, IL, March 31 to April 4, 2012.**
4. Cooper, R., Misra, S, Chaudhuri, M. and **Chaudhuri, G. (2012)** Physiological interaction of BRCA2 and PARP in the protozoan pathogen *Trypanosoma brucei.* **Presented at the Experimental Biology Annual Meeting at San Diego, April 20-April 25, 2012.**
5. Mittal, M. K., Misra, S. and **Chaudhuri, G. (2012)** Development of vitamin D-resistance in breast cancer cells through SLUG-mediated coordinate repression of CYP2R1, CYP27B1 and VDR gene promoters. **Presented at the Experimental Biology Annual Meeting at San Diego, April 20-April 25, 2012.**

6. Misra, S., Mittal, M. K., and **Chaudhuri, G. (2012)** ZAR2: a novel regulator of BRCA2 gene expression. **Presented at the 13th RCMI International Symposium on Health Disparities that convened December 9-13, 2012 in San Juan, Puerto Rico.**

7. Mittal, M. K., and **Chaudhuri, G. (2013)** Induction of apoptosis in the TNBC cells through dsDNA decoy-mediated functional impairment of SLUG activity. **Presented at the Ninth AACR-Japanese Cancer Association Joint Conference: Breakthroughs in Basic and Translational Cancer Research February 21-25, 2013, Hyatt Regency, Maui, Maui, HI.**

8. Mittal, M. K., Misra, S. and **Chaudhuri, G. (2013)** Increase in glutaminase (GLS1) levels through SLUG-induced repression of hsa-miR-23a in triple negative breast cancer cells. **Presented at the AACR Annual Meeting at Washington, DC, April 6-April 10, 2013.**

9. Mittal, M. K., and **Chaudhuri, G. (2013)** Induction of apoptosis in the TNBC cells through dsDNA decoy-mediated functional impairment of SLUG activity. **Presented at the Chromatin and Epigenetics in Cancer Meeting in Atlanta, GA on June 19-22, 2013.**

10. Misra, S., Mittal, M. K., Khedkar, S. and **Chaudhuri, G. (2013)** Methylation of BRCA2 gene promoter CpG units through ZAR2-dependent DNMT1 recruitment. **Presented at the Chromatin and Epigenetics in Cancer Meeting in Atlanta, GA on June 19-22, 2013.**

Conclusion:

We found that several genes that are involved in the activation/utilization of some of the common anti-breast cancer agents (e.g. anti-estrogens and vitamin D) are significantly repressed by the zinc finger transcriptional repressor SNAI2 in the aggressive and invasive breast tumors. These repression results in the development of resistance of the SNAI2-high breast tumor cells to those drugs. We also discovered that SNAI2 is a potent repressor of SNAI1 gene expression. Thus, often knockdown of SNAI2 results in elevation of the levels of SNAI1 and the effect of SNAI2 knockdown on its target genes are not fully appreciated. We have developed nucleic acid and peptide-based drugs against the functions of both of the SNAI proteins to combat against SNAI-high metastatic breast cancer along with vitamin D and anti-estrogens. ***We conclude that combinatorial treatment of SNAI-high breast tumor cells with the SNAI inhibitors will not only diminish their aggressiveness but also make these cells sensitive to the inhibition of some of the conventional anticancer agents such as vitamin D and anti-estrogens.***

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APPENDICES: None.